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Supporting Information

Sterilizing Immunity against SARS-CoV-2 Infection in Mice by a Single-Shot and Lipid Amphiphile Imidazoquinoline TLR7/8 Agonist-Adjuvanted Recombinant Spike Protein Vaccine**

Sonia Jangra[+], Jana De Vrieze[+], Angela Choi[+], Raveen Rathnasinghe, Gabriel Laghlali, Annemiek Uvyn, Simon Van Herck, Lutz Nuhn, Kim Deswarte, Zifu Zhong, Niek N. Sanders, Stefan Lienenklaus, Sunil A. David, Shirin Strohmeier, Fatima Amanat, Florian Krammer, Hamida Hammad, Bart N. Lambrecht, Lynda Coughlan, Adolfo García-Sastre, Bruno G. De Geest, and Michael Schotsaert**

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MATERIALS AND METHODS

Materials

All chemicals for synthesis were purchased from Sigma-Aldrich or TCI, unless noted otherwise. IMDQ was synthesized according to literature (1).

Mice, Cell lines and reagents

6-8 weeks old female BALB/c mice were obtained from Charles River Laboratories, MA and were housed in a specified pathogen-free facility at Icahn school of medicine at Mount Sinai, with food and water ad libitum, adhering to the guidelines from Institutional Animal Care and Use Committee. Ethical approval reference: IACUC-2017-0330.

Madin-Darby Canine Kidney Cells (MDCK, ATCC-CCL 34) and Vero-E6 (ATTC-CRL 1586, clone E6) cells are routinely cultured in the laboratory. Both cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal bovine serum (FBS) and additionally with 1% non-essential amino acids for Vero-E6 cells. DC2.4 mouse dendritic cells were cultured in RPMI-glutamax supplemented with 10 % fetal bovine serum (FBS), antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin) and 1 mM sodium pyruvate. Murine RAW blue 264.7 macrophages were cultured in DMEM medium supplemented with 10 % heat-inactivated FBS, antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin), 2 mM L-glutamine and 0.01 % Zeocin. Cells were incubated at 37 °C in a controlled and sterile environment of 95 % relative humidity and 5 % CO_2.

IMDQ-PEG-CHOL synthesis

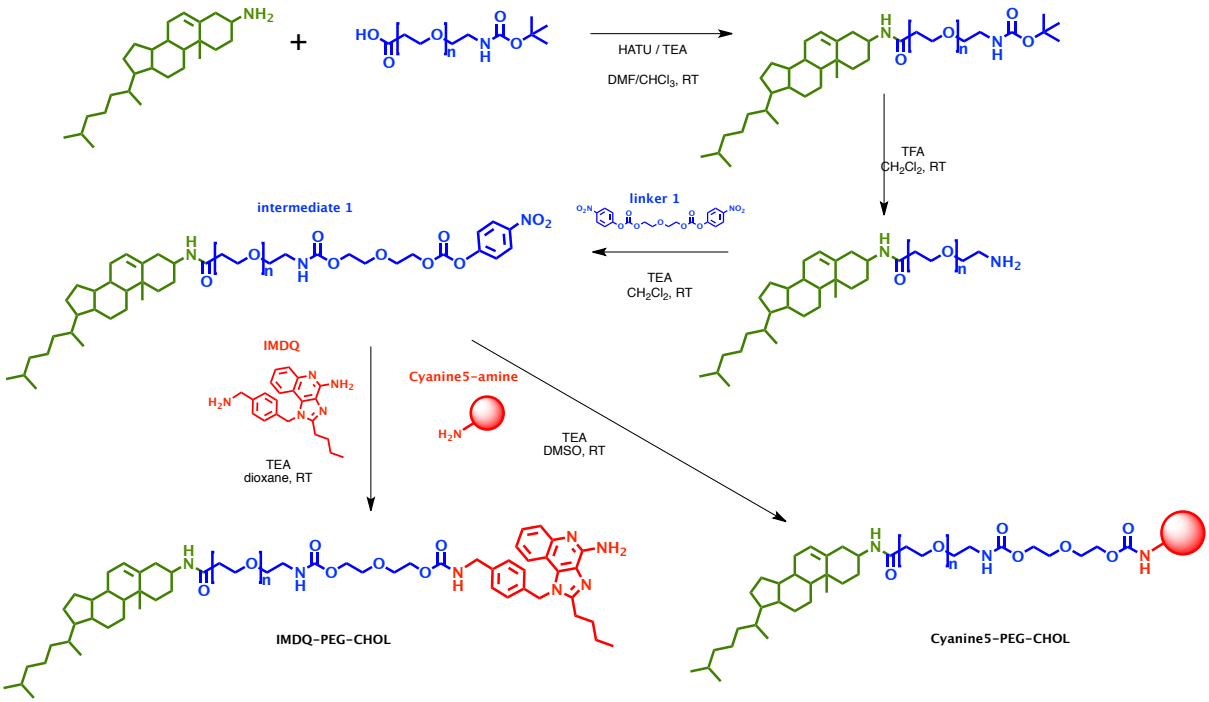
Synthesis of Cholesterylamine

First, the alcoholic hydroxyl group of cholesterol was transformed to an azide through a Mitsunobu reaction in the presence of diphenylphosphoryl azide (DPPA). Cholesterol (2.0 g, 5.17 mmol) was dissolved in a round bottom flask equipped with a stirring bar containing anhydrous THF (20 mL). Triphenylphosphine (PPh_3, 1.63 g, 6.21 mmol) and diisopropyl azodicarboxylate (DIAD, 1.22 mL, 6.21 mmol) was added to the round

bottom flask. Upon addition of DIAD, the reaction mixture developed a yellow colour. After 10 minutes, DPPA (1.34 mL, 6.21 mmol) was added and the mixture stirred overnight at room temperature under inert atmosphere. The reaction mixture was reduced under vacuum and further purified by column chromatography (cyclohexane), to yield a purified withe powder (yield = 60 %). The resulting product cholesteryl-N_3 was analysed by ^{1}H-NMR and ATR-IR.

Next, a Staudinger reduction was executed to reduce the azide group to a primary amine function with PPh_3. The obtained cholesteryl-N_3 (400 mg, 0.97 mmol) was transferred into a round bottom flask containing a stirring bar and dissolved in anhydrous THF (2.0 mL) under inert atmosphere. A solution of PPh_3 (2.55 g, 9.72 mmol) in dry THF (5.0 mL) was added. After 30 min, 2 mL water was added and the reaction mixture stirred overnight at room temperature equipped with a balloon to trap the released nitrogen gas. The reaction mixture was diluted extensively with toluene before being reduced under vacuum by 50 °C. The crude product was purified by column chromatography using a gradient (from 95:5 DCM:MeOH to 95:5 DCM:MeOH + 1% ammonium hydroxide), yielded a white powder which was characterized by ^{1}H-NMR, ATR-IR and MS(yield = 98 %).

ESI-MS (Figure SX): m/z $[M+H]^+$ = 386.37 (theoretical); found = 386.363



Scheme S1. Synthesis of IMDQ-PEG-CHOL and Cynanine5-PEG-CHOL.

*Synthesis of PEG-CHOL (**Scheme S1**)*

A round bottom flask containing a stirring bar was loaded with Boc-NH-PEG-COOH (300 mg, 0.098 mmol) and dissolved in anhydrous *N,N*-dimethylformamide (DMF, 3 mL) under inert atmosphere. 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) (40.84 mg, 0.107 mmol) was added to the stirring solution followed by 20.4 µL of triethylamine (TEA, 0.146 mmol). After 5 min, a solution of cholesterylamine (56.4 mg, 0.146 mmol) in dry chloroform (1.5 mL) was added and stirred at room temperature for 2 h, yielding a slightly yellow reaction mixture. The reaction mixture was co-evaporated with a large excess of toluene to remove DMF. Subsequently, the crude product was purified by threefold precipitation in ice cold diethyl ether followed by column chromatography (90:10 DCM: MeOH) (yield = 86 %). The white PEG-CHOL-NH Boc powder was analyzed by ^{1}H-NMR, DMAc-SEC and MALDI-ToF.

Subsequently the Boc-group was removed by dissolving Boc-NH-PEG-CHOL (200 mg, 0.058 mmol) in 2 mL of dichloromethane (DCM) in a round bottom flask equipped with a stirring bar. An equal amount of trifluoroacetic acid (TFA, 2 mL) was added and the solution was stirred for 2 h opened to ambient air and temperature. Prior to concentration under vacuum, a large excess of toluene was added to the reaction mixture. Finally, the product was transferred to dialysis membranes and dialyzed against 0.1 % v/v ammonium hydroxide solution in demineralized water for multiple days and one day against demineralized water. After lyophilization, the white fluffy powder NH_2-PEG-CHOL was characterized by ^{1}H-NMR and MALDI-ToF.

*Synthesis of **linker 1***

In a round bottom flask equipped with a stirring bar, p-nitrobenzyl chloroformate (2.02 g, 10 mmol) was dissolved in anhydrous DCM and cooled on ice. A mixture of diethylene glycol (424.5 mg, 4 mmol) and TEA (1.67 mL, 12 mmol) in 10 mL anhydrous DCM were added dropwise and stirred for an additional 30 minutes on ice. After 2 h on room temperature, the reaction mixture was concentrated under vacuum, dissolved in EtOAc and filtered. After evaporation of the solvent under reduced pressure, the linker was analyzed by ^{1}H-NMR and MS.

*Synthesis of IMDQ-PEG-CHOL (**Scheme S1**)*

First, **linker 1** (65.3 mg, 0.15 mmol) was dissolved in 2.5 mL anhydrous DCM under inert atmosphere. After addition of 2 equivalent of dry TEA (8.35 µL, 0.06 mmol), NH_2-PEG-CHOL (100 mg in 2 mL anhydrous DCM, 0.03 mmol) was added dropwise under stirring and a distinct yellow color appeared indicating the release of p-nitrophenol. After overnight reaction, purification was performed by double precipitation into a mixture of ice-cold hexane:acetone (80:20). The resulting **intermediate 1** was dried under vacuum and analyzed by NMR and SEC with DMAc as mobile phase.

In the second step, **intermediate 1** (50 mg, 0.014 mmol) and IMDQ (9.5 mg, 0.22 mmol) were weighed into a round bottom flask with stirring bar. The compounds were dissolved in 2.3 mL anhydrous 1,4-dioxane and anhydrous TEA (9.76 µL, 0.07 mmol) was added to solution under vigorous stirring. After 3 h at room temperature, a few drops of dry methanol were added and the reaction was stirred overnight. Next, the solution was transferred into a dialysis membrane (1 kDa) and dialyzed for multiple days against demineralized water. After freeze drying, the with fluffy*IMDQ-PEG-CHOL* powder was characterized by ^{1}H-NMR, HPLC and MALDI-ToF. HPLC conditions to verify absence of freely soluble IMDQ: LiChroCart® C18 column 250-4, mobile phase H_2O/ ACN 65:35 with 0.1% TFA, flow rate at 1 mL/min and detection at 250nm. Note that IMDQ-PEG was synthesized in similar fashion, but omitting the conjugation of cholesterylamine.

*Synthesis of Cyanine5-PEG-CHOL (**Scheme S1**)*

NH_2-PEG-CHOL (25 mg, 7.49 µmol) was weighted into a Schlenk tube equipped with a magnetic stirring bar and dissolved in 2.5 mL dry DMSO under inert atmosphere. Then, 0.21 mL of cyanine5 N-hydroxysuccinimide ester (stock solution of 25 mg/mL in anhydrous DMSO, 7.86 µmol) and anhydrous TEA (5.2 µL,37.42 µmol) were added to the Schlenk tube and further stirred overnight at room temperature. After dialyzed for three days against demineralized water, Cyanine5-PEG-CHOL was isolated as a fluffy blueish powder after lyophilization.

Biolayer interferometry analysis

Bovine serum albumin (BSA) was biotinylated by reacting it with 5:1 excess of biotin-NHS followed by dialysis and lyophilization. Hydrated streptavidin sensors were dipped in PBS to record a baseline for 60 seconds, followed by dipping into biotinylated BSA (12.5 nM, 66.5 kDa) in PBS for 300 s, and dipping for 30 s in PBS for washing. Next, a second baseline was recorded by dipping in fresh PBS for 120 s. Association of IMDQ-PEG-CHOL was measured by dipping into a solution of IMDQ-PEG-CHOL in PBS for 600 s. Note that the experiment was ran in parallel for different concentrations of IMDQ-PEG-CHOL. Dissociation of IMDQ-PEG-CHOL was recorded by dipping in PBs for 600 s. The experiment was performed in a black flat bottom 96 well plate set at 30 °C by 1000 rpm, using an Octet RED96 model (Pall Fortébio). Data processing was done by the FortéBio software package.

Cell cytotoxicity (MTT) assay

DC 2.4 cells were plated seeded in 96-well plates at a density of 8 000 cells per well in 200 µL culture medium. 50 µL of sample (dilution series in PBS, ranging from 10^{-4} mg/mL to 0.5 mg/mL), PBS (negative control, 100 % viability) and DMSO (positive control, 0 % viability) were added to the wells. After 72 h, the medium was aspirated and cells were washed with 200 µL PBS followed by addition of 100 µL of diluted MTT stock solution. After 1h, the solution was removed and the formed formazan crystals were dissolved in 50 µL DMSO. Quantification was done by measuring the absorbance at 590 nm using a microplate reader. Note, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 50 mg) was dissolved in 10 mL sterile PBS, filtrated (membrane 0.22 µm) and 1/5 diluted in culture medium prior to use in this assay.

Confocal microscopy

DC 2.4 cells were seeded in Willco-Dish glass bottom at a concentration of 10 000 cells in 180 µL culture medium and allowed to adhere overnight. Cells were pulsed overnight with 20 µL of a 1 mg/mL Cyanine5-PEG-CHOL or Cynanine5-PEG solution in PBS. Next, the culture medium was aspirated and cells were fixated with 4% paraformaldehyde (PFA) for 30 min followed by washing with PBS and confocal

imaging using a Leica DMI6000B microscope (63x 1.40 NA objective) coupled to an AndorDSD2 confocal scanner and a Zyla5.5 CMOS camera. Image processing was done using the ImageJ software package.

Flow cytometry analysis of in vitro IMDQ-PEG-CHOL association

DC 2.4 cells were seeded out in 24-well plate at a concentration of 200 000 cells per well in 450 µL of culture medium. Cells were pulsed with samples and incubated overnight at 37 °C. Afterwards, the supernatants were removed, cells were washed with PBS and detached with cell dissociation buffer (0.5 mL, 15 min, 37 °C). The content of the wells was transferred to an Eppendorf and centrifuged (5 min, 300 G, 4 °C). After aspiration of the supernatant, the cell pellets were resuspended in PBS and analyzed using a BD Accuri Flow Cytometer. Data were processed using the FlowJo software package.

RAW blue innate immune activation assay

RAW blue 264.7 macrophages were seeded in flat-bottom 96 well plate at a density of 70.000 cells per well, suspended in 180 µL culture medium and pulsed with 20 µL of sample for 24h at 37 °C at different concentrations of IMDQ-PEG-CHOL, IMDQ-PEG, IMDQ and PBS. Subsequently, 50 µL of supernatant was transfer to a new flat-bottom 96 well plate followed by addition of 150 µL of QUANTI-Blue™ reagent solution, prepared according to the manufacturer's instruction (Invivogen). After 30 minutes at 37 °C, the SEAP levels were determined by UV-Vis spectrophotometry at 620 nm using a microplate reader. Note, the colorimetric quantification of the samples was obtained relative to the negative control and each concentration was performed in fivefold.

Analysis of *in vivo* lymphatic drainage

20 µL (1 mg/mL in PBS) of Cyanine5-PEG-CHOL or Cyanine5-PEG were injected into the footpad of female C57BL/6 WT mice. Two mice were used per group and injected in both footpads. At the designated time point, mice where sacrificed and popliteal lymph nodes where isolated for flow cytometry and confocal imaging. A single cell suspension was prepared from the dissected popliteal lymph nodes for analysis by

flow cytometry. Isolated lymph nodes were collected in ice cold PBS, dissociated through 70 µm cell strainers, washed with PBS and stained with a fixable dead/live-staining. 123count ebeads were added to determine cellularity prior to analysis by a BD FACS Quanto flow cytometer. Data were processed using the FlowJo software package.

For confocal imaging popliteal lymph nodes where frozen in OCT cryomedium (Sakura, 4583). frozen sections (8-µm) were cut by cryostat. These sections where fixed for 4 min in PFA 2% (v/v), and washed with PBS. Images were acquired on a Zeiss LSM710 confocal microscope equipped with 488-nm, 561-nm and 633-nm lasers and with a tunable two-photon laser. Confocal imaging was done using a Leica DMI6000B microscope (10x 0.70 NA objective) coupled to an AndorDSD2 confocal scanner and a Zyla5.5 CMOS camera. Image processing was done using the ImageJ software package.

In vivo immune activation imaging

Luciferase reporter mice (IFNβ+/Δβ-luc) with a Balb/c background, aged 7-9 weeks, were housed in individual ventilated cages and given ad libitum access to food and water. 20 mL of IMDQ-PEG-CHOL, IMDQ-PEG or IMDQwere injected subcutaneously in the footpad (n=3) at an equivalent IMDQ dose of 2 mg. For in vivo imaging at the given time points, mice were injected subcutaneously with 200 µL D-luciferin and in vivo luminescence imaging was recorded 12 min later using the IVIS Lumina II imaging system. Local (DLN and DLN + foot pad) luminescence and full-body luminescence were quantified using the Living Image 4.4 software.

Analysis of *in vivo* lymphocyte targeting and activation

20 µL (containing an equivalent IMDQ dose of 2mg) of IMDQ-PEG-CHOL or Cyanine5-PEG-CHOL was injected into the footpad of female C57BL/6 WT mice. At the designated time point, mice where sacrificed and popliteal lymph nodes where isolated. A single cell suspension was prepared from the dissected popliteal lymph nodes for analysis by flow cytometery. Isolated lymph nodes were collected in ice cold PBS, smashed through 70 µm cell strainers, washed with PBS and stained for 30min

at 4°C with following primary labeled antibodies: CD3, CD20, CD11c, MHCII, CD86, CD40. Live dead ratio's where determined by staining with fixable dead/live- staining and 123count ebeads were added to determine cellularity prior to acquiring them on 123count ebeads were added to determine cellularity prior to Analysis by a BD FACS Quanto flow cytometer. Data were processed using the FlowJo software package.

Vaccines, Blood Collection and Serology

Quadrivalent inactivated influenza virus vaccine (QIV) was the human Seqirus vaccine (2018-2019 formula) containing the antigens of the following influenza virus strains: A/Singapore/GP1908/2015 IVR-180 (H1N1), A/North Carolina/04/2016 (H3N2), B/Iowa/06/2017 and B/Singapore/INFTT-16-0610/2016. Vaccine was obtained from BEI resources and mixed with adjuvant as described below. Vaccine was injected once via the intramuscular route with a BD 300 µL insulin syringe in the hamstring muscles of the both hind legs (50 µL/leg). The administered vaccine dose corresponds to 1.5 µg of each hemagglutinin type in the vaccine per mouse.

Blood was collected twenty days post vaccination via submandibular bleeding and serum was prepared by allowing the blood to clot at room temperature. Anti-HA antibody responses were measured by enzyme linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) assay. For quantification of HA-specific total IgG levels by ELISA, 96 well NUNC Maxisorp plates were coated with QIV (2 µg/ml HA equivalent for each HA) in bicarbonate buffer at 4 °C overnight. After washing and blocking with 4% milk for 1 h at room temperature, serum samples 3-fold diluted starting at 1/100 in PBS with 0.05% Tween20 were allowed to bind ELISA antigen for 1.5 h at room temperature. Plates were washed three times with PBS (0.05% Tween20) and incubated with sheep-derived anti-mouse total IgG (GE Healthcare, Amersham, UK), IgG1 or IgG2a (Invitrogen) serum conjugated to horse-radish peroxidase. After a final washing step, tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, San Diego, CA, USA) was used to estimate levels of HA-specific mouse IgG by measuring the OD_{450} with the OD_{650} as a reference after stopping the colorimetric reaction with 1M H_2SO_4.

Hemagglutination inhibition was performed as previously described (2). Briefly, four volumes of receptor destroying enzyme (RDE, Vibrio cholera filtrate, Sigma Aldrich,

San Diego, CA, USA) were added to each volume of mouse serum. After overnight incubation at 37 °C, sera were heat-inactivated at 56 °C for 30 min in sodium citrate buffer. Four hemagglutination units of IVR-180 H1N1 virus were mixed with twofold dilutions of treated sera in a final volume of 50 µL. Mixtures of virus and diluted serum were allowed to bind for 1h at room temperature before 50 µL of 0.5% chicken red blood cell suspension was added. HI titers were read after 1h incubation on ice.

Trimeric recombinant SARS-CoV-2 spike protein was produced as previously described: only the ectodomain of the spike protein (GenBank: MN908947.3) was cloned into a mammalian expression plasmid and the cleavage site was removed and stabilizing prolines were added at position 986 and 987 (3–5). A hexa-histidine tag as well as a T4 foldon trimerization domain was present in the plasmid for ease of purification. The spike protein was expressed in 293F cells, using the ExpiFectamine 293 Transfection Kit (Thermo Fisher). Supernatant was collected on day 3 post transfection and Ni-NTA agarose (Qiagen) was used to purify the protein. This protocol has been described in much greater detail earlier (5). Vaccine (6 µg/mouse) was mixed with adjuvant as described below and injected once via the intramuscular route with a BD 300 µL insulin syringe in the hamstring muscles of both hind legs (50 µL/leg).

Anti-SARS-CoV-2 spike protein ELISA was performed to estimate spike-specific antibody responses upon vaccination. Briefly, maxisorp Nunc 96-well microtiter plates were coated with 50 µl per well of recombinant spike protein, diluted to a concentration of 2 µg/ml in carbonate/bicarbonate buffer and incubated overnight at 4°C. Three-fold serially diluted serum samples, starting from 1:100, were added to the antigen-coated plates followed by overnight incubation at 4°C. The plates were then washed in 1X PBS + 0.01% Tween20 and again incubated with appropriate horse-radish peroxidase (HRP)-conjugated secondary antibodies targeting total IgG, IgG1 or IgG2a antibodies (GE Healthcare). The plates were washed and developed with 50 µl of TMB substrate per well until blue color appeared. The reaction was terminated with 50 µl 1M H_2SO_4 and the absorbance was measured at 450nm with 650 nm as a reference.

In vitro microneutralization assay

To measure the neutralizing potential of SARS-CoV-2 vaccine-induced sera, an *in vitro* microneutralization assay was performed similar to the protocol described in (6). Briefly, the Spike ± adjuvant-vaccinated mice sera were inactivated at 56°C for 30 min.

Serum samples were serially diluted 2-fold starting from 1:10 dilution in infection medium (DMEM+ 2% FBS+ 1X non-essential amino acids). The samples were incubated with 100 tissue culture infective dose 50 (TCID50) which equals 40 plaque forming units (PFU) of SARS-CoV2 virus for 1 hour in an incubator at 37°C, 5% CO_2 and then transfer on pre-seeded Vero-E6 cells in 96-well cell-culture plates. The plates were incubated at 37°C for 48 hours and fixed in 4% formaldehyde. The cells were washed with 1XPBS and blocked in 5% milk in 1XPBS+ 0.1% Tween20 for 1 hour at room temperature. After blocking, the cells were permeabilized with 0.1% TritonX100, washed and incubated with anti-SARS-CoV-2-nucleoprotein and anti-SARS-CoV-2-Spike monoclonal antibodies, mixed in 1:1 ratio, for 1.5 hours at room temperature. The cells were washed again and incubated with HRP-conjugated anti-mouse IgG secondary antibody for 1 hour at room temperature followed by a brief PBS wash. Finally, 50 μl tetramethyl benzidine (TMB) substrate was added and incubated until blue color appeared and the reaction was terminated with 50 μl 1M H_2SO_4. Absorbance at 450nm was recorded and percentage inhibition calculated. Anti-mouse SARS-CoV-2-nucleoprotein and anti-mouse SARS-CoV-2-Spike antibodies were obtained from the Center for Therapeutic Antibody Development at the Icahn School of Medicine at Mount Sinai, New York.

Adjuvants: AddaVax was purchased from Invivogen and mixed at a 3:1 ratio vaccine:AddaVax per the manufacturer's recommendation. IMDQ adjuvants were mixed with vaccine at an equivalent of 10 μg core IMDQ (100 μg of IMDQ-PEG-CHOL, see below) per mouse.

Viruses and Infection

QIV vaccinated mice were infected 24 days post vaccination with 100 lethal dose 50 (18,000 PFU) of egg-grown influenza IVR-180 H1N1 virus, a vaccine strain that contains the surface antigens of influenza A/Singapore/gp1908/2015 (H1N1) virus. Morbidity and mortality were monitored for eight days. A group of age-matched naïve animals was added to the experiment to confirm the dose of virus was lethal to unvaccinated animals.

In order to make SARS-CoV-2 Spike-vaccinated BALB/c mice susceptible to challenge with wild type SARS-CoV-2 virus, airway expression of human ACE-2, the receptor for

SARS-CoV-2, was obtained by intranasal transduction of mice with $2.5x10^8$ PFU of adenovirus expressing h-ACE-2 (Ad5-hACE2), 4.5-weeks post-vaccination as described in (7). Five days after transduction with Ad5-hACE2, mice were challenged with $5x10^4$ PFU of SARS-CoV2 isolate USA-WA1/2020 (BEI resources; NR-52281) per mice. Body weights were recorded to assess the morbidity during the days post challenge.

Lung Virus Titration

Plaque assays were performed to quantify and compare the lung viral titers in vaccinated versus unvaccinated mice. As described previously (2), whole lungs were harvested from the mice and homogenized in 1 ml 1XPBS. After brief centrifugation, the tissue debris was discarded and the supernatant was 10-fold serially diluted starting from 1:10 dilution. For IVR-180, MDCK cells were incubated with the lung homogenate dilutions for 1 hour at 37°C, 5% CO_2 and then overlaid with a mixture of 2% oxoid agar and 2X minimal essential medium (MEM) supplemented with 1% diethyl-aminoethyl (DEAE)-dextran and 1 µg/ml tosylamide-2-phenylethyl chloro-methyl ketone (TPCK)-treated trypsin. After 48 hours of incubation at 37°C, 5% CO_2, the plates were fixed in 4% formaldehyde and immune-stained with IVR-180-post-challenge polyclonal serum. Similarly, For SARS-CoV-2, pre-seeded Vero-E6 cells were incubated with diluted lung homogenates for 1 hour at room temperature and then overlayed with a 1ml mixture of 2% oxoid agar and 2X MEM supplemented with 2% FBS. After 72 hours of incubation at 37°C, 5% CO_2, the plates were fixed in 4% formaldehyde, followed by immune-staining of infected cells with anti-mouse SARS-CoV-2 nucleoprotein and anti-mouse SARS-CoV-2 spike monoclonal antibodies. After incubation in primary antibodies, HRP-conjugated anti-mouse secondary antibody was added for 1 hour. Finally, the plaques were developed with TrueBlue substrate (KPL-Seracare). The final viral titers were calculated in terms of plaque forming units (PFU)/ml.

References

1 Shukla NM, Malladi SS, Mutz CA, Balakrishna R, David SA. Structure−ActivityRelationships in Human Toll-Like Receptor 7-Active Imidazoquinoline Analogues. J Med Chem. 2010 Jun 10;53(11):4450–65.

2. Choi A, Ibañez LI, Strohmeier S, Krammer F, García-Sastre A, Schotsaert M. Non-sterilizing, Infection-Permissive Vaccination With Inactivated Influenza Virus Vaccine Reshapes Subsequent Virus Infection-Induced Protective Heterosubtypic Immunity From Cellular to Humoral Cross-Reactive Immune Responses. Front Immunol [Internet]. 2020 Jun 9 [cited 2020 Aug 9];11. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7296151/

3. Amanat F, Stadlbauer D, Strohmeier S, Nguyen THO, Chromikova V, McMahon M, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans. Nat Med. 2020;26(7):1033–6.

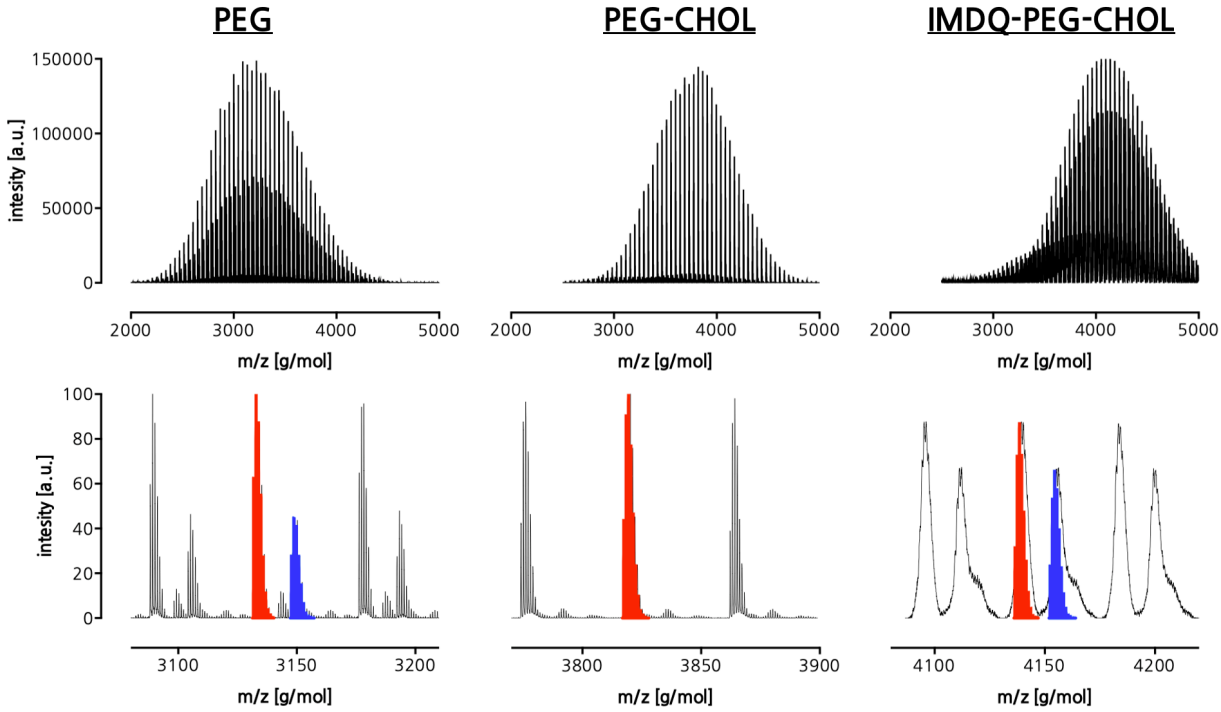
4. Margine I, Palese P, Krammer F. Expression of functional recombinant hemagglutinin and neuraminidase proteins from the novel H7N9 influenza virus using the baculovirus expression system. J Vis Exp JoVE. 2013 Nov 6;(81):e51112.

5. Stadlbauer D, Amanat F, Chromikova V, Jiang K, Strohmeier S, Arunkumar GA, et al. SARS-CoV-2 Seroconversion in Humans: A Detailed Protocol for a Serological Assay, Antigen Production, and Test Setup. Curr Protoc Microbiol. 2020;57(1):e100.

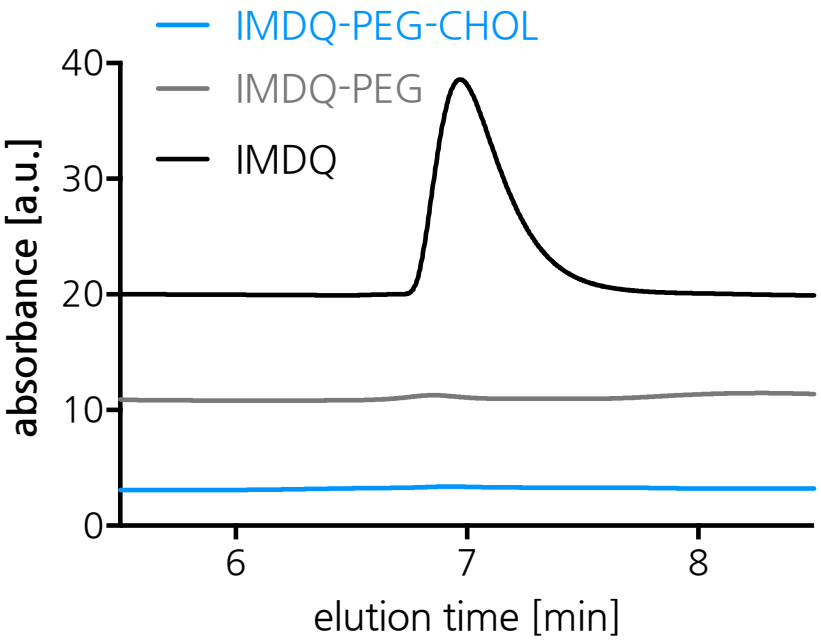
6. Amanat F, White KM, Miorin L, Strohmeier S, McMahon M, Meade P, et al. An In Vitro Microneutralization Assay for SARS-CoV-2 Serology and Drug Screening. Curr Protoc Microbiol [Internet]. 2020 Sep [cited 2020 Oct 2];58(1). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7361222/

7. Sun J, Zhuang Z, Zheng J, Li K, Wong RL-Y, Liu D, et al. Generation of a Broadly Useful Model for COVID-19 Pathogenesis, Vaccination, and Treatment. Cell. 2020 Aug 6;182(3):734-743.e5.

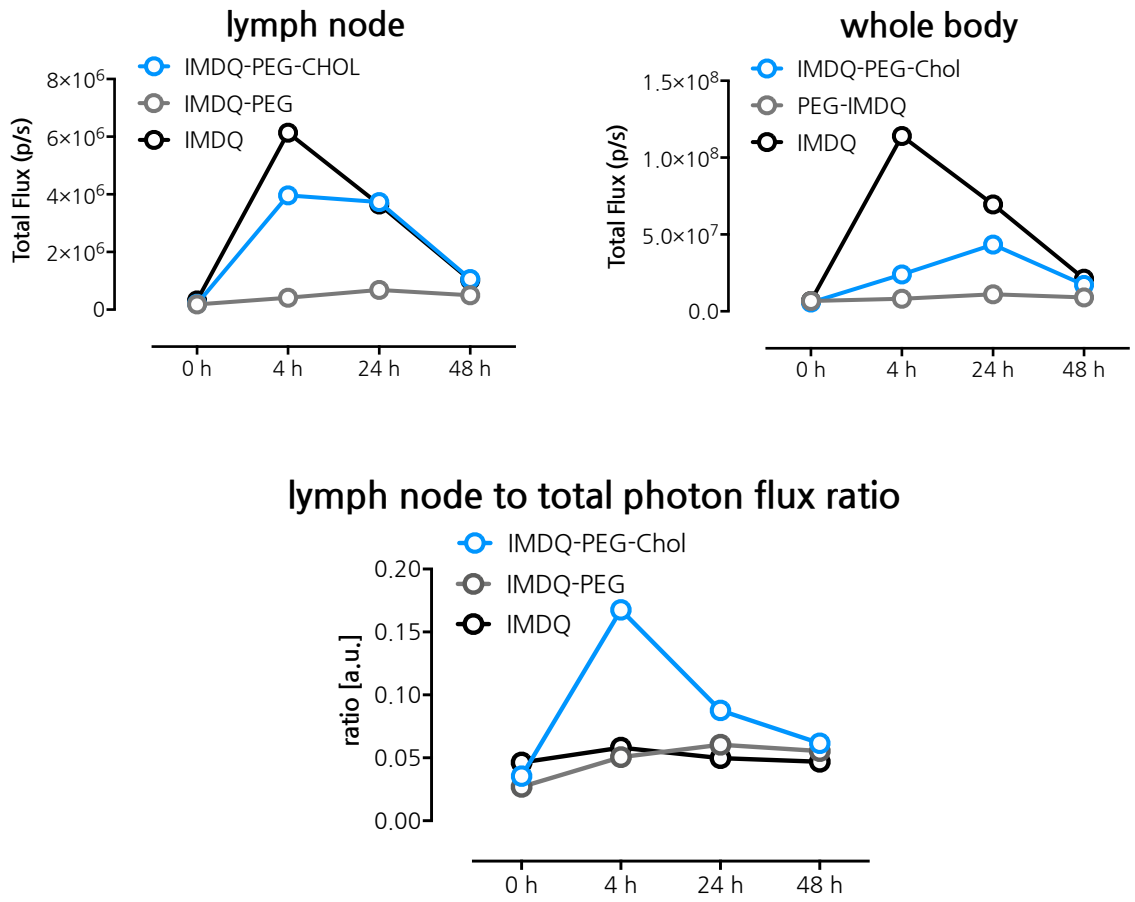
SUPPLEMENTARY FIGURES



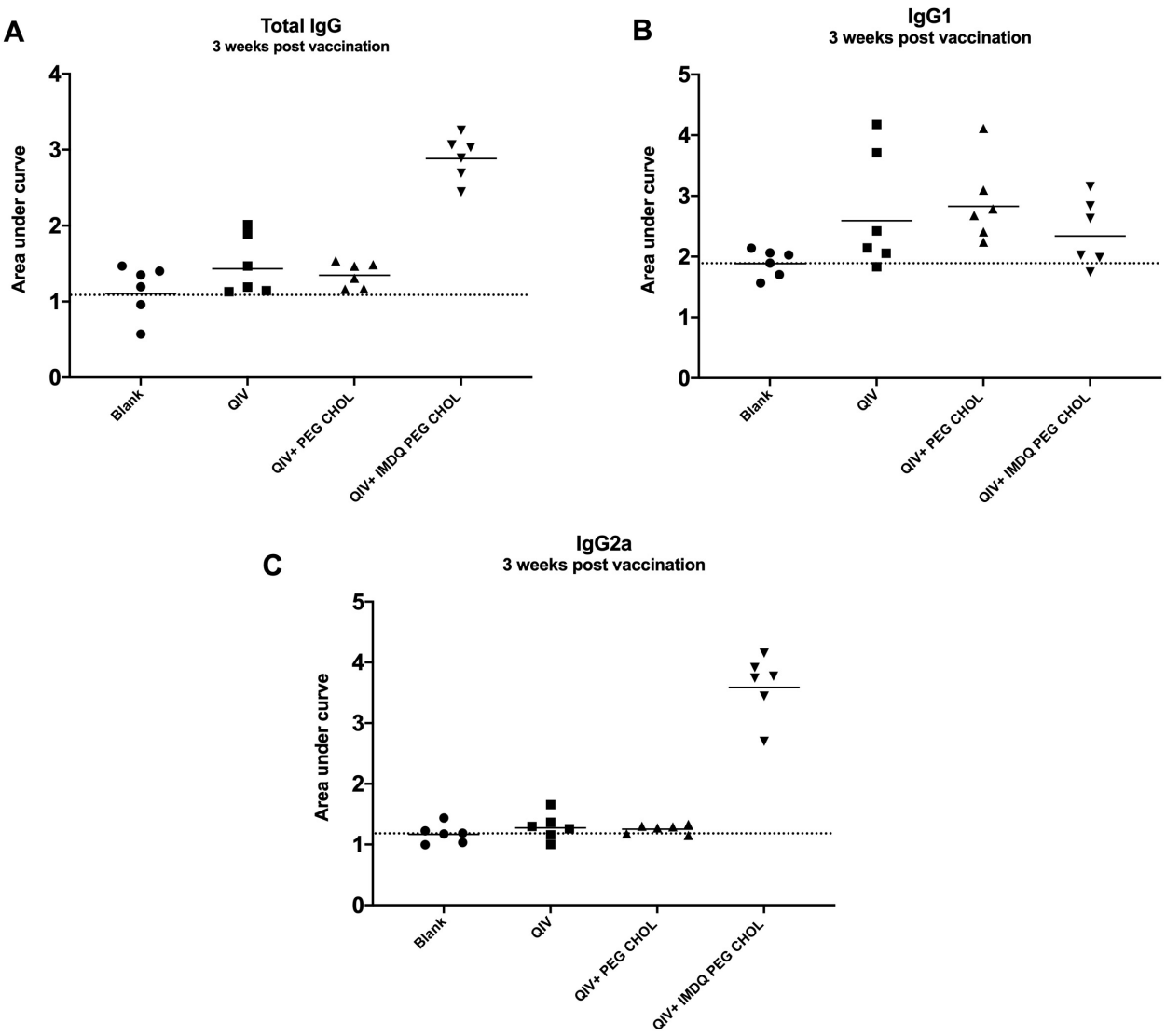
Supplementary Figure S1: MALDI-ToF analysis of PEG, PEG-Chol and IMDQ-PEG-CHOL. The bottom row depicts a zoom highlighting the simulated sodium adduct in red and the potassium adduct in blue.



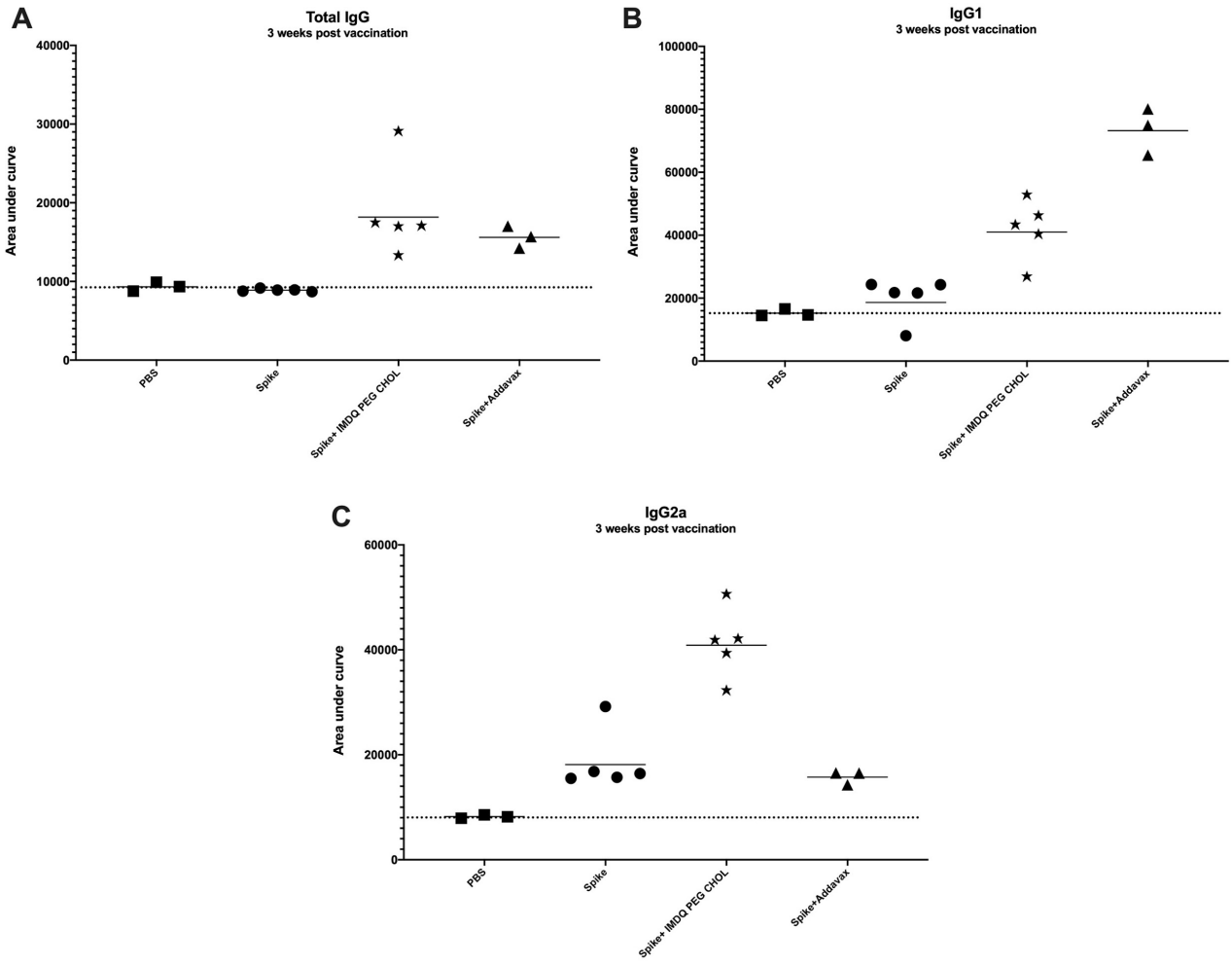
Supplementary Figure S2: HPLC elugrams (eluens 50:50acetonitrile/water with 0.1 vol%TFA) showing absence of unmodified IMDQ in IMDQ-PEG and IMDQ-PEG-CHOL as no IMDQ peak (emerging at 7 min in the IMDQ elugram (black curve)) is observed in the IMDQ-PEG and IMDQ-PEG-CHOL elugrams (blue and grey curves).



Supplementary Figure S3: Quantification of the total photon flux from the draining popliteal lymph node and the whole body, and the ratio of both. Quantification was done based on the bioluminescence images (**Figure 3A**) of luciferase reporter mice (IFNβ+/Δβ-luc) recorded 4, 24 and 48 h post footpad injection of IMDQ-PEG-CHOL, IMDQ-PEG and native IMDQ



Supplementary Figure S4: ELISA titers for total IgG, IgG1 and IgG2a in mice sera collected 3 weeks post-vaccination. The ratio IgG2a/IgG1 is representative of the Area under the OD450 curve of the individual serum sample.



Supplementary Figure S5: ELISA titers for total IgG, IgG1 and IgG2a in mice sera collected 3 weeks post-vaccination. The ratio IgG2a/IgG1 is representative of the Area under the OD450 curve of the individual serum sample.